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Phylogenetic diversity of Moroccan cork oak woodlands fungi

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Interspecific variation among 87 sporocarps of fungi belonging to 15 genera and 39 species were evaluated by analyzing the internal transcribed spacer (ITS) of the rDNA region using restriction fragment length polymorphism (RFLP). The ITS region was first amplified by polymerase chain reaction (PCR) with specific primers and then cleaved with different restriction enzymes. Amplification products, which ranged between 500 and 950 base pairs (bp), were obtained for all the isolates analyzed. The degree of polymorphism observed did not allow proper identification of most of the species. Cleavage of amplified fragments with the restriction enzymes *Alu* I, *EcoR* I and *Hinf* I revealed extensive polymorphism. The fifteen genera and most species presented specific restriction patterns. The only species not identifiable by a specific pattern belonged to the genera *Russula* (*R. decipiens* and *R. straminea*). These species might be considered as closely related species. The *Pisolithus* sporocarps had two ITS-RFLP types with one dominating. ITS sequencing confirms that the two RFLP types correspond to two distinct species of *Pisolithus*. Our data show the potential of ITS region PCR-RFLP for the molecular characterization of ectomycorrhizal fungi and their identification and monitoring in artificial inoculation programs.
Keywords. Diversity, fungi, Internal Transcribed Spacer (ITS), ribosomal DNA (rDNA), PCR-RFLP, Morocco.

Diversité phylogénétique des champignons des subéraies marocaines. La variabilité interspécifique de 87 carpophores des champignons appartenant à 15 genres et 39 espèces ont été évaluées par l'analyse du polymorphisme de longueur de fragments de restriction (RFLP) de l'espaceur interne transcrit (ITS). Cette région a été d'abord amplifiée, avec des amorces spécifiques, par la réaction de polymérisation en chaine (PCR), puis digérée avec différentes enzymes de restriction. Un produit d'amplification, dont la taille variait entre 500 et 950 paires de bases (pb), a été obtenu pour tous les isolats analysés. Le degré de polymorphisme observé ne permet pas l'identification de la plupart des espèces. L'analyse RFLP, avec les enzymes de restriction *Alu* I, *EcoR* I et *Hinf* I, des fragments amplifiés révèle un grand polymorphisme. Les quinze genres et la plupart des espèces présentent des profils de restriction spécifiques. Les deux espèces du genre *Russula* (*R. decipiens* et *R. straminea*) présentent le même profil de restriction. Ces espèces pourraient être considérées comme étroitement apparentées. Les carpophores de *Pisolithus* montrent deux profils ITS-RFLP distincts. Le séquençage de l'ITS confirme que ces deux profils correspondent à deux espèces distinctes de *Pisolithus*. Nos données montrent l'importance de l'analyse PCR-RFLP de l'ITS pour la caractérisation moléculaire et l'identification des champignons ectomycorhiziens et leur suivi dans les programmes d'inoculation artificielle.
Mots-clés. Diversité, champignon, espaceur interne transcrit (ITS), ADN ribosomique (ADNr), PCR-RFLP, Maroc.

1. INTRODUCTION

In Morocco, the cork oak woodlands of Maâmora forest undergo a worrying regression in spite of the intensive plantation programs. The success of these programs with a good plant establishment after transfer to the field, is related among others to the cultural techniques used in nurseries. Ectomycorrhizal

symbiosis, a mutualistic plant-fungus association, plays a fundamental role in the biology and ecology of forest trees, affecting growth, water and nutrient absorption, and providing protection from root diseases (Smith et al., 1997). The controlled mycorrhization in nurseries, by selected ectomycorrhizal fungi, improves survival, establishment, and growth of seedlings after out planting (*e.g.* Marx, 1977; Le Tacon et al., 1992). It is estimated that between 5,000 and 6,000 species of fungi worldwide form ectomycorrhizas (Molina et al., 1992). Most of these are basidiomycetes, but a small number of ascomycetes and zygomycetes are also known to produce this type of symbiosis (Smith et al., 1997). Phenotypic differences between two isolates of the same species of an ectomycorrhizal fungus may be as pronounced as the differences between two distinct species, and for this reason identification of these fungi is not clear-cut (De la Bastide et al., 1995). Aboveground surveys of sporocarps are usually poor indicators of the community structure below ground (Arnolds, 1991; Jonsson et al., 2000; Horton et al., 2001). This is because sporocarp production is triggered by specific environmental conditions (Gardes et al., 1991a; Eggers, 1995). A classical approach for identifying mycorrhizas is therefore to trace hyphal connections between sporocarps and mycorrhizal sheaths (Agerer, 1995). However, special skills are required when root density is so high that hyphae emerging from the stipe base cannot be attributed unambiguously to a single mycorrhizal type (Pritsch, 1996). An alternative promising way to identify mycorrhizas consists of comparing specific DNA regions of mycorrhizas and sporocarps. Polymerase chain reaction coupled with restriction fragment length polymorphism analyses (PCR/RFLP) have been applied in mycorrhizal research to identify strains of introduced or naturally occurring mycorrhizal fungi (Gardes et al., 1991a; 1993; Henrion et al., 1992; 1994a), or of economically important species such as *Tuber* (Henrion et al., 1994b), and also to differentiate and identify mycorrhizal symbionts unambiguously (Erland et al., 1994; Kraigher et al., 1995; Gardes et al., 1996a). In these studies, two target sequences within the ribosomal DNA, ITS (Internal Transcribed Spacer) and IGS (Intergenic Spacer), have mainly been used to detect polymorphism between and within species of ectomycorrhizal taxa. The ITS region separating genes 17S and 25S can be amplified by specific primers anchored in these two units. Since the ITS region is highly conserved intraspecifically but variable between different species it is often used in taxonomy (Bruns et al., 1991; Hillis et al., 1991), but ITS region polymorphism of identifying ectomycorrhizal fungi species has been determined for only a restricted number of species, leaving its full potential as a taxonomic tool as yet unexplored (Karén et al., 1997). The PCR/RFLP polymorphism of the ITS region is generally regarded as appropriate to differentiate at the species level (Eggers, 1995; Gardes et al., 1996b). Martin et al. (1998), based on PCR/RFLP of the rDNA ITS, have been set up an expandable database of DNA profiles and fingerprints of ectomycorrhizal fungi in a World Wide Web (WWW) Internet Server.

In this study, 87 sporocarps of known fungi belonging to 15 genera and 39 different species were analyzed using the PCR/RFLP technique as applied to the ITS regions of these fungi. These analyses aimed to confirm the classification of these fungi and to elucidate their interspecies biodiversity. The ultimate aim of our investigation is to establish a database of DNA profiles of Moroccan cork oak woodlands ectomycorrhizal fungi to help identification of different morphotypes found in the field.

2. MATERIALS AND METHODS

2.1. Sporocarp collections

Sporocarps were collected from eight different Moroccan cork oak woodlands all located at the northern part of Morocco. Data are presented only for well identified ones according to distinct macroscopic and microscopic characters (**Table 1**).

2.2. Isolates cultivation

Pure cultures were obtained by transferring aseptically pieces of fruit bodies on the Modified Melin Norkrans (MNM) agar medium (Marx et al., 1975). They were incubated on the culture medium in the dark for 25 days at 28°C. All the isolates are conserved within the INRA (Montpellier, France) and the Forest Research Centre (Rabat, Morocco) ectomycorrhizal fungi culture collections, on MNM under 2°C.

2.3. Total DNA extraction and PCR amplification of ITS rDNA

Total DNA was extracted from fruiting bodies using the CTAB protocol (Gardes et al., 1993; Henrion et al., 1994b) or the DNeasy Plant Mini Kit according to the manufacturer's recommendations (QIAgen SA). The ITS of the rDNA was amplified by using ITS1 and ITS4 primers (White et al., 1990). PCR amplification was carried out using a PCR-100 thermocycler (MJ Research, Inc. Watertown, MA, USA) using 50 µL reaction volumes each containing: 1 µL DNA template, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer and 0.6 units of *Taq* DNA polymerase (Eurogentec, Belgium). The amplification conditions were 1 cycle of 95°C for 5 min, followed by 35 cycles including 94°C for 30 s,

Table 1. Fungi studied, number of sporocarps sampled and their origin — *Champignons étudiés, nombre de sporophores échantillonnés et leur origine*.

Sporocarp			
	Name	**Number**	**Origin**
1	*Amanita crocea*	1	Lalla Mimouna
2	*Amanita pantherina*	1	Lalla Mimouna
3	*Amanita vaginata*	1	Dardara 2
4	*Boletus aereus*	16	Tanakoube 2, Maâmora
5	*Boletus fragrans*	1	Tanakoube 2
6	*Clitocybe odora*	1	Dardara 1
7	*Clitopilus prunulus1*	3	Tanakoube 1
8	*Clitopilus prunulus 2*	3	Tanakoube 2
9	*Entoloma lividum*	1	Tanakoube 1
10	*Inocybe asterospora*	1	Tanakoube 1
11	*Inocybe fastigiata*	1	Tanakoube 1
12	*Lactarius chrysorrheus*	1	Moulay Abdessalam
13	*Lactarius decipiens*	3	Moulay Abdessalam
14	*Lactarius fulvissimus*	1	Tanakoube 1
15	*Lactarius rugatus*	1	Lalla Mimouna
16	*Lactarius serifluus*	1	Tanakoube 2
17	*Lyophyllum lanzonii*	1	Dardara 2
18	*Pisolithus spp.*	19	Sidi Kahlifa, Mâamora
19	*Russula amoenolens*	2	Lalla Mimouna
20	*Russula carminipes*	1	Tanakoube 2
21	*Russula chlorides*	1	Tanakoube 1
22	*Russula cyanoxantha*	1	Tanakoube 2
23	*Russula decipiens*	3	M. Abdessalam, Mâamora
24	*Russula faustiana*	1	Tanakoube 2
25	*Russula fuliginosa*	1	Tanakoube 2
26	*Russula graveolens*	1	Maâmora
27	*Russula lepida*	1	Dardara 2
28	*Russula lepidicolor*	1	Tanakoube 1
29	*Russula luteotacta*	1	Tanakoube 1
30	*Russula odorata*	1	Tanakoube 2
31	*Russula praetervisa*	1	Tanakoube 1
32	*Russula straminea*	3	L. Mimouna, Mâamora
33	*Russula vesca*	1	Tanakoube 2
34	*Scleroderma meridionale*	3	Kenitra
35	*Sowerbyella rhenana*	1	Tanakoube 2
36	*Telephora terrestris*	3	Maâmora
37	*Tricholoma ustale*	1	Tanakoube 1
38	*Xerocomus cisalpinus*	1	Tanakoube 2
39	*Xerocomus ferrugineus*	1	Tanakoube 1

55°C for 30 s, 72°C for 1 min with a final extension at 72°C for 7 min. Negative controls (no DNA template) were included in all PCR experiments to check for DNA contamination.

2.4. RFLP analysis of ITS diversity

Per each 10 µL restriction digest, 8 µL amplified ITS product was mixed with the appropriate restriction buffer and 5 units of the appropriate endonucleases *Alu* I, *EcoR* I and *Hinf* I (Gibco BRL, Life Technologies) and then incubated for at least 2 h at 37°C. ITS and digested ITS products were respectively migrated by electrophoresis on 2% and on 3% regular (Sigma) and Nusieve (FMC) agarose gels, stained with ethidium bromide and photographed using the Oncor-Appligene Imager 2.02. Digested 100 bp (molecular weight marker, Boehringer Mannhein) and Smart ladder (Eurogentec, Belgium) were used as ladder.

2.5. Data analysis

After restriction with endonucleases, the amplification products were scored as 1 (presence) or 0 (absence) of a restriction site and used for determining genetic distances between the isolates (Nei et al., 1979). These distances were used to cluster the isolates by the unweighted pair group method with averages (UPGMA) method using the Statistica program (version 4.5 for Windows, StatSoft, Inc. 1993, Tulsa, OK, USA).

3. RESULTS

A total of 87 sporocarps in 15 genera and 39 species were analyzed, with a range of 1-3 sporocarps of all the species except for *Pisolithus* spp. and *Boletus aereus* represented by 19 and 16 sporocarps, respectively. PCR amplification with specific primers for the ITS region generated bands ranging from 500 to 950 bp. **Figure 1** shows that all samples presented only one amplification product. In general, no difference was detected between species from the same genus. To detect a wider range of polymorphism, the PCR products were cleaved with the restriction enzymes *Alu* I, *EcoR* I and *Hinf* I. The *Alu* I restriction enzyme produced 39 ITS-RFLP

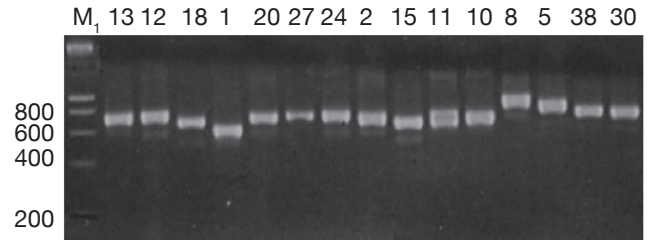

Figure 1. Gel electrophoresis of the amplified rDNA internal transcribed spacer (ITS) region of some fungal sporocarps — *Électrophorèse sur gel des produits d'amplification de l'ADN ribosomique de l'espaceur interne transcrit (ITS) de certains sporophores des champignons*.

Lanes are numbered according to the sporocarp identification scheme given in **table 2** — *les numéros des individus sont identiques à ceux décrits dans le* ***tableau 2***; M_1: size marker — *marqueur de poids moléculaire* (Smart ladder).

patterns including those of *Pisolithus* spp., while *EcoR* I and *Hinf* I generated 35 and 34 patterns, respectively. The restriction fragment sizes of ITS digested with these respective enzymes are summarized in **table 2** and examples of ITS-RFLP profiles are depicted in **figure 2a**, **2b** and **2c**. All the species showed polymorphic patterns, except two species of *Russula* (*R. decipiens* and *R. straminea*) which showed the same RFLP type for the three endonucleases. For this reason, the PCR products from these *Russula* species were further cleaved with two other enzymes (*Hae* III, *Taq* I) but still no polymorphism was detected. Furthermore, all the sporocarps of the same species (*e.g. Boletus aereus*, *Russula amoenolens*, *R. decipiens* and *R. straminea*) collected at local and large scale, except *Pisolithus* spp. and *Clitopilus prunulus*, yielded a single RFLP type. By using the enzymes *Alu* I, *EcoR* I and *Hinf* I, two patterns have been observed within *Pisolithus* and *Clitopilus prunulus* sporocarps; only one of these restriction enzymes is enough to elucidate this polymorphism. A comparison of *Pisolithus* ITS sequences using the BLAST program showed that

Table 2. Restriction fragments sizes of ITS regions of the studied fungi digested with *Alu* I, *EcoR* I and *Hinf* I — *Taille des fragments de restriction de la région ITS des champignons étudiés, obtenue avec les enzymes de restriction* Alu *I,* EcoR *I et* Hinf *I.*

	Species	ITS band sizes (bp)			
		Uncut	***Alu* I**	***EcoR* I**	***Hinf* I**
1	*Amanita crocea*	604	150, 65	550	290, 200,110
2	*Amanita pantherina*	780	300, 290	410, 370	240, 170
3	*Amanita vaginata*	700	440	510	310
4	*Boletus aereus*	850	150, 80	550, 300	260, 100, 80
5	*Boletus fragrans*	830	120, 80	490, 340	340, 240, 170
6	*Clitocybe odora*	825	290, 177	335	295
7	*Clitopilus prunulus* 1	900	470, 180	335, 300	340
8	*Clitopilus prunulus* 2	825	540, 180	310	260
9	*Entoloma lividum*	850	420, 380	350, 240	360, 260
10	*Inocybe asterospora*	720	300	410	340, 200, 170
11	*Inocybe fastigiata*	670	290	370	340, 310
12	*Lactarius chrysorrheus*	750	290	340, 320	340, 240
13	*Lactarius decipiens*	750	290, 190, 65	340, 320	340, 240
14	*Lactarius fulvissinus*	800	500, 200	400, 300	440, 360
15	*Lactarius rugatus*	720	290, 120, 80	360, 320	240, 200, 140
16	*Lactarius serifluus*	750	440, 180	400, 280	440, 295
17	*Lyophyllum lanzonii*	860	560, 300	335	460, 400
18	*Pisolithus arhizus*	650	190, 120	650	240, 170, 130, 110
19	*Pisolithus* sp. 4	650	123, 148	184, 232	330, 270
20	*Russula amoenolens*	760	290	400, 360	310
21	*Russula carminipes*	800	500, 240	335, 250	295, 260
22	*Russula chlorides*	850	500	400, 280	440, 260
23	*Russula cyanoxantha*	830	500, 150	335, 280	340
24	*Russula decipiens*	770	440, 230	490, 280	340, 290
25	*Russula faustiana*	800	300	340, 100	300, 200
26	*Russula fuliginosa*	850	440, 160	335, 260	340
27	*Russula graveolens*	770	300, 325	490, 280	340, 290
28	*Russula lepida*	950	390, 270	370, 260	410, 340
29	*Russula lepidicolor*	750	390, 260	400, 280	400, 300
30	*Russula luteotacta*	855	440, 290	490, 340	375, 340
31	*Russula odorata*	740	470, 270	400, 280	400, 300
32	*Russula praetervisa*	850	560, 280	370, 280	340, 260
33	*Russula straminea*	770	440, 230	490, 280	340, 290
34	*Russula vesca*	740	500	335, 280	380, 295
35	*Scleroderma meridionale*	790	400, 290	640	230
36	*Sowerbyella rhenana*	500	500	340	300, 180
37	*Telephora terrestris*	700	120, 170, 260	290, 320	340, 290
38	*Tricholoma ustale*	870	290, 150,120,65	430	375
39	*Xerocomus cisalpinus*	880	560	400, 260	380, 260, 170
40	*Xerocomus ferrugineus*	860	540, 160	730	380, 295

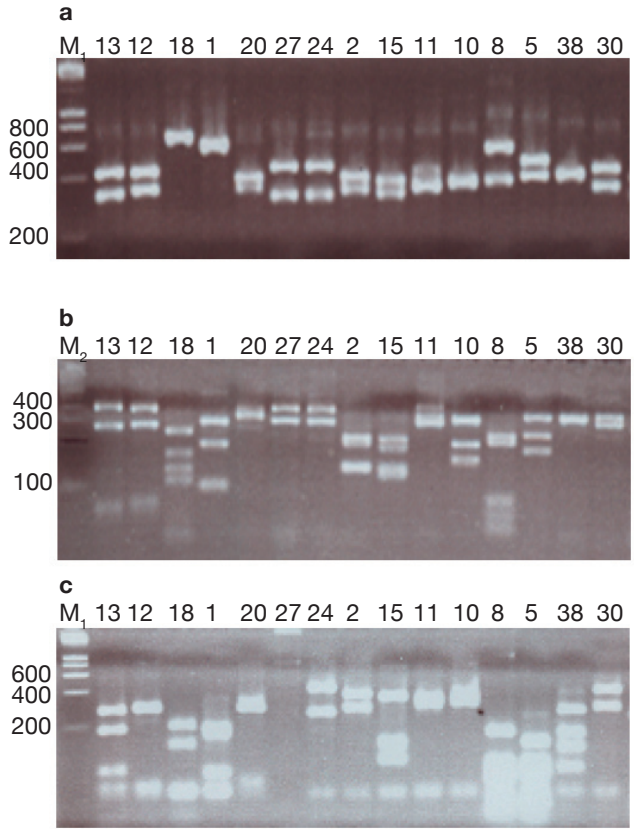

Figure 2. Gel electrophoresis of the amplified rDNA internal transcribed spacer (ITS) region of some fungal sporocarps digested with the *EcoR* I (**a**), *Hinf* I (**b**) and *Alu* I (**c**) restriction enzymes — *Électrophorèse sur gel des produits d'amplification de l'ADN ribosomique de l'espaceur interne transcrit (ITS) de certains sporophores des champignons digérés avec les enzymes de restriction* EcoR *I* (***a***), Hinf *I* (***b***) *et* Alu *I* (***c***).

Lanes are numbered according to the isolate identification scheme given in **table 2** — *les numéros des individus sont identiques à ceux décrits dans le* ***tableau 2***; M_1 and M_2: size markers, Smart and 100 bp ladders respectively — *marqueurs de poids moléculaire respectivement Smart ladder et 100 pb ladder.*

the ITS-RFLP type 1 and type 2 were respectively very similar (96-98% of sequence similarities) to ITS of species 6 and species 4 defined by Martin et al. (2002). The ITS-RFLP type 1 was the dominant pattern representing 97% of collected sporocarps. The neighbor-joining tree separated the isolates into two groups (data not shown). Group I contained ITS-RFLP type 1 from *Pisolithus arrhizus* named species 6, while group II contained ITS-RFLP type 2 from *Pisolithus* sp. named species 4 (Martin et al., 2002). Restriction fragments obtained with all the endonucleases tested were used to determine genetic distances between the genotypes and cluster them into specific groups. The phylogenetic tree constructed is depicted in **figure 3**. In general, no genera specific group was detected. Except for *R. decipiens* and *R. straminea* which considered as closely related species and some species of the same genera which formed specific group, most of the species were clustered independently of their belonging to a genus.

4. DISCUSSION

Mycorrhizal inoculation of seedlings with selected ectomycorrhizal fungi is now applied worldwide to improve the survival, establishment and growth of seedlings after out planting (Le Tacon et al., 1992; Grove et al., 1993). Morphological descriptions (Agerer, 1991) or allozymes (Sen, 1990) have provided useful data for identifying the mycorrhizal fungi below ground, but most species have not been described by these methods. Today a wide range of molecular techniques can be used to detect DNA sequence variation in ectomycorrhizal fungi (Gardes et al., 1991a; 1991b; Henrion et al., 1992; Bruns et al., 1993).

Detection of polymorphism using PCR-RFLP analyses of the rDNA ITS region has been successfully used for identifying several species of fungi (Amicucci et al., 1996; Gardes et al., 1996a; Di Battista, 1997; Karen et al., 1997; Pritsch et al., 1997). This simple technique requires only minute amounts of DNA and two specific primers flanking the ITS region. We found that the amplification products for the ITS region of 39 species (100%) of fungi collected in Moroccan cork oak woodlands ranging from 500 to 950 bp, coincided with the sizes obtained for the other fungi (Gardes et al., 1991a; Karen et al., 1997; Martin et al., 1998). Despite the length polymorphism observed for many of the species, ITS analysis alone was not able to separate all the genotypes. RFLP analysis of the ITS region has been suggested by several authors as a means for discriminating between fungi at the interspecific and intraspecific level (Gardes et al., 1990; 1991b; Bruns et al., 1991; Manassila et al., 2005; Guerin-Laguette, 1998). Cleavage of the ITS region with *Alu* I (**Figure 2**) allowed differentiation of 40 out of the 39 identified species according to macroscopic and microscopic characteristics (**Table 2**). In fact, two ITS-RFLP types of *Pisolithus* are identified and ITS sequence analysis indicated that the two ITS-RFLP types correspond to two distinct *Pisolithus* species, species 6 and species 4, recognized by Martin et al. (2002). Furthermore, none of the restriction enzymes produced a distinct pattern for the 2 *Russula* species (*R. decipiens* and *R. straminea*). Henrion et al. (1992) considered those types of species, which do not differ enough from the ITS region to be distinguished with this marker (*e.g. Laccaria bicolor* and *Laccaria laccata*) as closely related species.

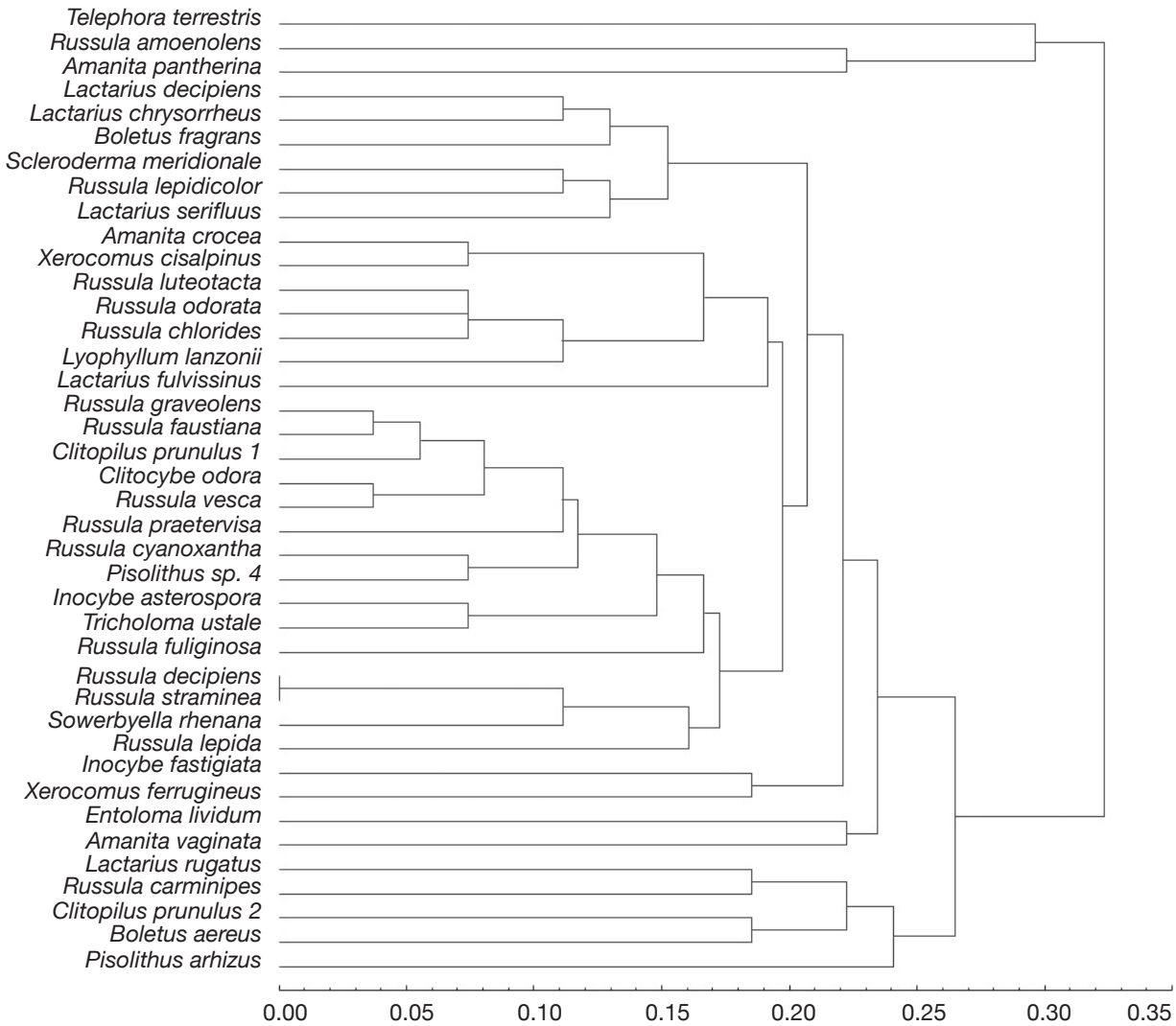

Figure 3. Unweighted pair group method with averages (UPGMA) cluster diagram of the relationships between 40 fungi species given in **table 2** — *Dendrogramme généré par UPGMA des relations génétiques entre les 40 espèces de champignons décrites dans le* ***tableau 2***.

A correlation between soil properties and diversity within the genus *Pisolithus* have been revealed (Bakkali Yakhlef et al., 2007; 2008). Due to this high degree of interspecific variation of the ITS, a matching of RFLP types of mycorrhizas and identified sporocarps (reference) RFLP types within the same geographical area will be likely to indicate identical species. Basing on PCR/RFLP of ITS of ectomycorrhizal fungi, a RFLP database has been setting up in a World Wide Web Internet server (Martin et al. 1998).

Community studies of ectomycorrhizal fungi are based either on identification of mycorrhizas (the so-called below ground view), or on monitoring of fruit body production (above-ground view) (Richard et al., 2005). It is clear that fruit body surveys reveal the presence of ECM taxa in a fast and inexpensive way (Richard et al., 2004). However these studies often underestimate the presence of numerous taxa, like resupinate and hypogeous fungi and taxa lacking an apparent sexual stage (Horton et al., 2001). *Cenococcum geophilum*, for example, which is an abundant, cosmopolitan species lacking sexual structures and reproduces via sclerotia and/or mycelia (Jonsson et al., 2000; Horton et al., 2001), has been found in abundance, below ground, in cork oak woodlands (Abourouh, 1991).

The results outlined in this paper show that interspecific variation of the ectomycorrhizal fungi ITS region is relatively high. ITS restriction fragment analysis has potential for developing species-level markers for many, but not necessarily all, ectomycorrhizal fungi species. It appears that ITS-RFLP is a potent tool for the taxonomic study of ectomycorrhizal fungi, with the minute amounts of DNA required and the high reproducibility of this procedure making it an ideal

method both for studying population heterogeneity in the field and the identification and monitoring of specific strains introduced into the soil in controlled mycorrhization programs.

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